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Efficient Surface Sterilant for Establishment of Dendrobium *in vitro* Culture

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ABSTRACT

Background: Most orchid species in the Dendrobium genus are epiphytic. Dendrobium orchid (*Dendrobium nobile* L.) var. Sonia is a non-woody epiphytic orchid that bears a beautiful purple-coloured flower and grows in soilless medium. Dendrobium can be propagated vegetatively through divisions or Keikis. But this propagation method is extremely slow. Micropropagation is most suitable method for multiplication of dendrobium orchids. Surface sterilization is an important step in the preparation of vigorous and viable explants in micropropagation. Most of the surface contaminants can be abolished by surface sterilization with a suitable sterilizing agent. The aim of the investigation is to present an effective disinfection method for the aseptic culture of Dendrobium var. Sonia by using nodal segments.

Methods: In the present investigation, two sterilant were employed namely mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) at different concentrations and durations. A total of nine combinations were tried for treating nodal explants in this investigation during 2022-2023. Explants were surface sterilized with 70% ethanol for 5 minutes followed by 3 to 4 washes with sterile distilled water. Thereafter, the explants were subjected to different surface sterilization treatments to establish contamination free cultures.

Result: The study revealed that among the 9 treatments, minimum contamination (30.40%) was recorded in 0.1% $HgCl_2$ for 10 minutes which helps in the maximum establishment of healthy culture (69.60%). Whereas, the lowest result for establishment was found in autoclaved distilled water with maximum contamination (100%). Maximum explant mortality (20.80%) due to sterilant was observed in NaOCl (2%) for 20 minutes.

Key words: Aseptic culture, Contamination, *Dendrobium nobile*, Explant, Mercuric chloride, Sterilants.

INTRODUCTION

Orchids are flowering plants, that belong to the orchidaceae family. Orchids are popularly grown for their wide range of colours *i.e.,* red, pink, white, blue, green, purple, orange and yellow and a diverse variety with long vase life (Toukuhara and Mii, 2001). Orchidaceae is 2nd largest family after Asteraceae. Some of the important genera are *Bulbophyllum*, *Epidendrum*, *Dendrobium* and *Pleurothallis.* Dendrobium is one of the important and 2nd largest genera (Vendrame and Carvalho, 2008). Some of the species that are indigenous to India are *D. aphyllum, D. transparens, D. densiflorum, D. fimbriatum and D. nobile.* It is perennial, has a non-woody structure and is epiphytic. *Dendrobium nobile* is a purple-coloured flower with a white colour at the center. It blooms in spring and winter. It is propagated vegetatively through keikis, shoot tip (Saiprasad and Polisetty, 2002), node (Pathania *et al.,* 1998) and tissue culture. Tissue culture is becoming the most efficient and reliable method for the mass production of true to type healthy and disease-free plantlets (Martin *et al.,* 2003; Çimen and Özge, 2009 and Raad *et al.,* 2011).

The explants such as node, pseudobulb, leaf tips, *etc.* can be used for *in vitro* multiplication. It can multiply directly (organogenesis) or indirectly (callogenesis) by culturing the explants. Basal medium is supplemented through various plant growth regulators (such as auxin and cytokinin) at different concentrations which helps in the growth and development of the plantlet. Many problems were faced during the establishment such as phenolic oxidation (browning caused by the oxidation of phenolic compound), microbial contamination and minimum inoculation density. For tissue culture operations to be successful, aseptic (free of all micro organisms) or sterile

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conditions must be maintained. Every culture vessel, medium and tool used in handling must be kept aseptic. Every procedure needs to be done in a sterile, laminar airflow cabinet (Chawala, 2003). The explant itself and its tissues need to be disinfected *i.e.*, surface sterilization of explant. The main goal of disinfection techniques is to strike a balance between lowering infection and promoting explant survival and regeneration, both of which are greatly affected by the disinfectant employed and the physiological state of the explants (Traore *et al.,* 2005); Jan *et al.,* 2013). The source, size and age of explants are some of the components that affect the success of disinfection (Silva *et al.,* 2016). Plant material so collected should be healthy and disease free. The size of the explants can affect the penetration of disinfectants and the efficiency of surface sterilization (Hall, 1999). In terms of the age of planting material, older plants may have dormant pathogens and therefore make disinfecting more challenging (Silva, 2013), Silva and Dobránszki, 2013). Along with this pretreatment of donor plants also plays a major role in reducing the cause of contamination (Silva *et al.,* 2016). To eradicate the fungal or bacterial contamination, various methods and procedures have been developed (Hussain,1994; Herman, 1996). The rate of contamination can reduce the survival percentage, reduce multiplication and can lead to mortality. It is very challenging to achieve the planting material contamination free (Mihaljevic *et al.,* 2013). Sterilization includes treatment with chemical solutions like mercuric chloride, sodium hypochlorite and ethanol (George, 1993). Since the plant tissues are also harmed by these sterilizing agents, the right concentration of sterilant, the length of time exposed to the sterilant and the application sequences of these sterilant must be standardized to reduce explant damage and improve survival (CPRI, 1992). Sterilant concentration can vary according to the planting material, species type, growth environment and age of the plant (Mihaljevic *et al.,* 2013). Therefore, to control the problems of microbial contamination a protocol is necessary to be developed. Keeping this objective, the present investigation aims to establish an effective disinfection method for aseptic culture and increase the survival percentage of the explant of Dendrobium var. Sonia by using nodal segments.

MATERIALS AND METHODS

The experiment was conducted at Tissue Culture Laboratory, BAC, Bihar Agricultural University (BAU Communication No. 1525/231003), Sabour, Bhagalpur, Bihar, India during the 2022-2023.

Collection of explants

The explants were collected from healthy plants of Dendrobium var. Sonia is grown and maintained at polyhouse complex, Bihar Agricultural University, Sabour. The orchid beds should be fumigated at least 24 hours before the collection of explant node and pseudobulb of age 1.5-2.0 months were selected as explant. Orchid plants were fumigated before 24 hours of explant collection. With the help of a sterile surgical blade, the explants were incised and collected in a sterile bottle. The explants were collected from healthy and disease free plants.

Cleaning and pre-treatment of explant

Collected explants were cleaned and incised into small segments (keeping at least one node in each segment) and kept in running water approximately for 30 minutes to remove all the adhering dust particles and then to sterile water wash. Then it was treated with fungicide (Mancozeb 2.0 g/l) and 2-3 drops of Tween-20 solution for 45 minutes with continuous agitation with the help of a shaker. Then it was given 2-3 sterile water wash followed by antibiotic treatment (0.1% Streptomycin sulphate) for 30 minutes and agitated continuously. And finally, rinse through sterile water (2-3 wash). Rest procedures were given in Laminar Air Flow Cabinet *i.e.*, in a sterile condition.

Explant surface sterilization

The UV light of laminar air flow should be kept on for 45 minutes before use. The glassware (autoclaved) taken inside and the laminar air flow cabinet should be thoroughly wiped with 70% ethanol. The explants were rinsed with 2-3 wash of sterile water. There after, the explants were submerged in 70% ethanol for 5 minutes. Again given 3 sterile water wash. Followed by sterilant treatment. Sterilant *viz*. mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) were used at different concentrations for varying time durations (Table 1). The explants were given a final sterile water wash for 3 times and now the explants were ready to be inoculated. The explants were incised in 1.0 to 1.5 cm onto autoclaved sheets with the help of a heat sterilized scalpel. Then the explants were inoculated in prepared MS media by holding the bottle near to burner (carrying out all the procedures near the burner). Further, the inoculated bottles were capped and sealed with polyethylene film and marking the date of inoculation on the top of the cap and kept the bottles in the incubation room in a controlled condition.

RESULTS AND DISCUSSION

This investigation revealed that, significantly low percentage of contamination (30.40%) was observed in nodal segment

Table 1: Sterilant concentration and duration of treatment.

Treatment	Sterilant concentration and duration of treatment
T_0	Autoclaved distilled water
T_1	$HgCl_2$ (0.1%) for 5 min
T_2	$HgCl_2$ (0.1%) for 10 min
T_3	$HgCl_2$ (0.2%) for 5 min
T_4	$HgCl_2$ (0.2%) for 10 min
T_5	NaOCl (2%) for 10 min
T_6	NaOCl (2%) for 20 min
T_7	NaOCl (4%) for 10 min
T_8	NaOCl (4%) for 20 min

explants, surface sterilized with the treatment T_2 (0.1% $HgCl_2$ for 10 minutes) followed by treatment T_6 (2% NaOCl for 20 minutes) with 32.80% contamination. Whereas, maximum contamination percent was observed in treatment T0 (autoclaved water). Contamination-free culture is an essential condition for successful *in vitro* regeneration. The growth of explants is dependent on the type of sterilant used, its concentration and for the time duration it is treated. The maximum part of the contamination was reduced from mancozeb treatment at 0.2%. In terms of the highest contamination-free explants (69.60%) were observed in treatment T_2 (0.1 % $HgCl_2$ for 10 minutes) with zero mortality followed by treatment T_6 *i.e.*, 2% NaOCl for 20 minutes (67.20%) and lowest contaminated free culture was obtained at autoclaved water (Table 2). Significantly maximum aseptic culture (69.60%) was found in T_2 (0.1% $HgCl_2$ for 10 minutes) followed by treatment T_6 *i.e.*, 2% NaOCl for 20 minutes (67.20). All cultures were found contaminated at treatment T_0 *i.e.*, untreated control (Fig 1).

Contamination free culture is an essential condition for successful *in vitro* regeneration. The growth of explants is dependent on the type of sterilant used, their concentration and the time duration it is treated. The maximum part of the contamination was reduced from fungicide treatment *i.e.*, to 0.2% Mancozeb and further reduced from sterilant used and the best result came out to be at 0.1% $HgCl_2$ at 10 minutes. Similar results were obtained, where 80% of survivability was obtained at 0.1% of $HgCl_2$ for 10 minutes and 70% ethanol (Parvaty, 2022). Maximum culture establishment (85.67%) was observed when explants were sterilized with 0.1% $HgCl_2$ for 3 minutes+ 70% C_2H_6O for 30 seconds in Anthurium (*Anthurium andraeanum* Lind.) cv. Jewel by Thokchom and Maitra, (2016). In *Gerbera jamesonii* cv. Rosalin, maximum survivability (64.89%) was found at the combination of 0.1%

Table 2: Efficient surface sterilant for establishment of Dendrobium *in vitro* culture.

Treatment	Contamination %	Contamination free%	Aseptic culture%	Explants mortality due to sterilant	Culture establishment
T_0 (Autoclaved water)	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_1 ($HgCl_2$ 0.1% 5 min)	67.20 (55.08)	32.80 (34.92)	32.80 (34.92)	0.00 (0.00)	32.80 (34.92)
T_2 ($HgCl_2$ 0.1% 10 min)	30.40 (33.43)	69.60 (56.57)	69.60 (56.57)	0.00 (0.00)	69.60 (56.57)
T_3 ($HgCl_2$ 0.2% 5 min)	45.60 (42.47)	54.40 (47.53)	54.40 (47.53)	8.00 (16.22)	46.40 (42.93)
T_4 ($HgCl_2$ 0.2% 10 min)	59.20 (50.31)	40.80 (39.69)	40.80 (39.69)	12.00 (20.16)	28.80 (32.39)
T_5 (NaOCl 2% 10 min)	76.80 (61.25)	23.20 (28.75)	23.20 (28.75)	8.00 (16.01)	15.20 (22.75)
T_6 (NaOCl 2% 20 min)	32.80 (34.90)	67.20 (55.10)	67.20 (55.10)	20.80 (27.08)	46.40 (42.93)
T_7 (NaOCl 4% 10 min)	49.60 (44.77)	50.40 (45.23)	50.40 (45.23)	20.00 (26.52)	30.40 (33.41)
T_8 (NaOCl 4% 20 min)	73.60 (59.15)	26.40 (30.85)	26.40 (30.85)	12.00 (20.16)	14.40 (22.08)
SE(m)±	1.00	1.00	1.00	1.07	1.30
CD at 5%	2.85	2.85	2.85	3.03	3.69
CV (%)	4.29	5.97	5.97	17.05	9.10

Note- Values in the parenthesis are arc sine transformed data.

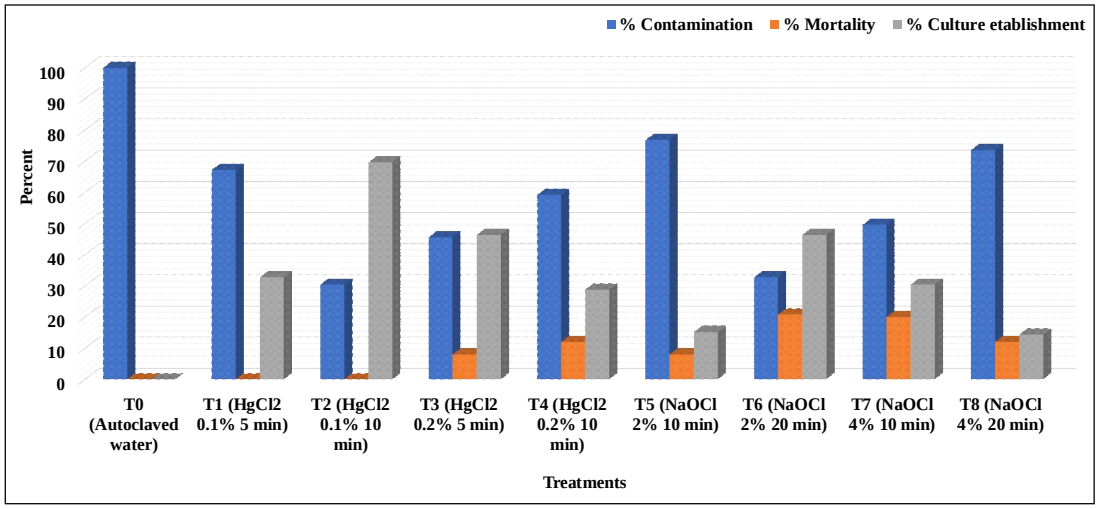

Fig 1: Effect of surface sterilant for establishment of *Dendrobium nobile* under *in vitro* conditions.

$HgCl_2$ for 1 minute and ethyl alcohol (70%) for 30 seconds by Thokchom and Maitra (2017). Ahamed and Ferdaous (2019) found 40% contamination free explants on 0.1% $HgCl_2$ for 6 minutes in *Gerbera jamesonii* Bolus. Whereas, Balilashaki *et al.* (2014) observed only 10% contamination at 7% sodium hypochlorite for 10 minutes in *Phalaenopsis amabilis* cv. Cool 'Breeze'. Abubakar and Pudake (2019) reported the result of sterilization procedures, revealed 15% NaHClO3 (5 min) + 70% ethanol (30s) + 0.1% $HgCl_2$ (5min) to be the most effective in controlling contamination in *Curcuma caesia* among all the treatments studied, with 85% contaminants free cultures after 16 days of inoculation. Maximum mortality was observed at treatment T_6 *i.e.*, 2% NaOCl for 20 minutes (20.80) which resulted in 46.40% of culture establishment. However, the least mortality (0%) was observed at 0.1% $HgCl_2$ at 5 and 10 minutes. A reduced mortality percentage (11.33), with the least contamination percent (3.67) at 0.1% $HgCl_2$ for 3 minutes + 70% C_2H_6O for 30 seconds observed by Thokchom and Maitra (2016). Sharma and Beura (2021) observed 6% mortality at 0.1% $HgCl_2$ solution for 4-7 minutes followed by 0.5% NaOCl solutions for 2 minutes. Maximum culture establishment or survival percent (69.60) was obtained at 0.1% of $HgCl_2$ for 10 minutes. Maximum aseptic culture per cent (96.67) with maximum survivability (96.67%) in *Phalaenopsis hybrids* cv. Shagan was also reported by Madhuri *et al.* (2021). The seed treated with 0.1% $HgCl_2$ within 6 to 15 minutes showed that the survival rate increased from 34.29% to 75% in Red Lotus (Trang *et al.,* 2021). In shoot culture of *Dendrobium aurantiacum* maximum sterilization efficiency was found at 20% NaOCl for 15 minutes which gives a 73.3±5.8% survival rate after 1 month of culture by Ma *et al.* (2020).

CONCLUSION

Overcoming the problem of poor sterilization effect of Dendrobium orchid caused by prolonged field-planting time and abundant phenolic substances in plants found in previous preliminary experiments, the aseptic system of node segments of Dendrobium var. Sonia was established, which is helpful to the efficient propagation of excellent individual plants. The successful establishment of the aseptic system is the basis for *in vitro* regeneration of plants, which provides a guarantee for successful tissue culture of Dendrobium. From the present investigation, it can be concluded that surface sterilization is a very crucial step for the maximum rate of survivability or establishment of culture. And best and desired results in terms of minimum contamination with maximum establishment can be obtained when the explants are treated with 0.1% $HgCl_2$ for 10 minutes.

Conflict of interest

The authors declare that they have no competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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